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In silico and *in vivo* analysis reveal a novel gene in *Saccharomyces cerevisiae* trehalose metabolism

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Abstract

Background: The ability to respond rapidly to fluctuations in environmental changes is decisive for cell survival. Under these conditions trehalose has an essential protective function and its concentration increases in response to enhanced expression of trehalose synthase genes, *TPS1*, *TPS2*, *TPS3* and *TSL1*. Intriguingly, the *NTH1* gene, which encodes neutral trehalase, is highly expressed at the same time. We have previously shown that trehalase remains in its inactive non-phosphorylated form by the action of an endogenous inhibitor. Recently, a comprehensive two-hybrid analysis revealed a 41-kDa protein encoded by the *YLR270w* ORF, which interacts with NTH1p.

Results: In this work we investigate the correlation of this Trehalase Associated Protein, in trehalase activity regulation. The neutral trehalase activity in the *ylr270w* mutant strain was about 4-fold higher than in the control strain. After *in vitro* activation by PKA the *ylr270w* mutant total trehalase activity increased 3-fold when compared to a control strain. The expression of the *NTH1* gene promoter fused to the heterologous reporter *lacZ* gene was evaluated. The mutant strain lacking *YLR270w* exhibited a 2-fold increase in the *NTH1-lacZ* basal expression when compared to the wild type strain.

Conclusions: These results strongly indicate a central role for Ylr270p in inhibiting trehalase activity, as well as in the regulation of its expression preventing a wasteful futile cycle of synthesis-degradation of trehalose.

Background

In the yeast *Saccharomyces cerevisiae*, cytosolic trehalose is mobilized by hydrolysis to glucose catalyzed by neutral trehalase (EC 3.2.1.28), encoded by the *NTH1* gene. NTH1p was shown to be post-translationally regulated by two different mechanisms: i) phosphorylation by cAMP dependent protein kinase (PKA) that activates the enzyme [1,2], ii) by an inhibitory protein [3]. Trehalase activity varies during growth on glucose as the result of the phosphorylation state of the enzyme [4]. At the onset of diauxie, activity undergoes a drastic decrease [4], in contrast to the high level of its mRNA [7] and the constant

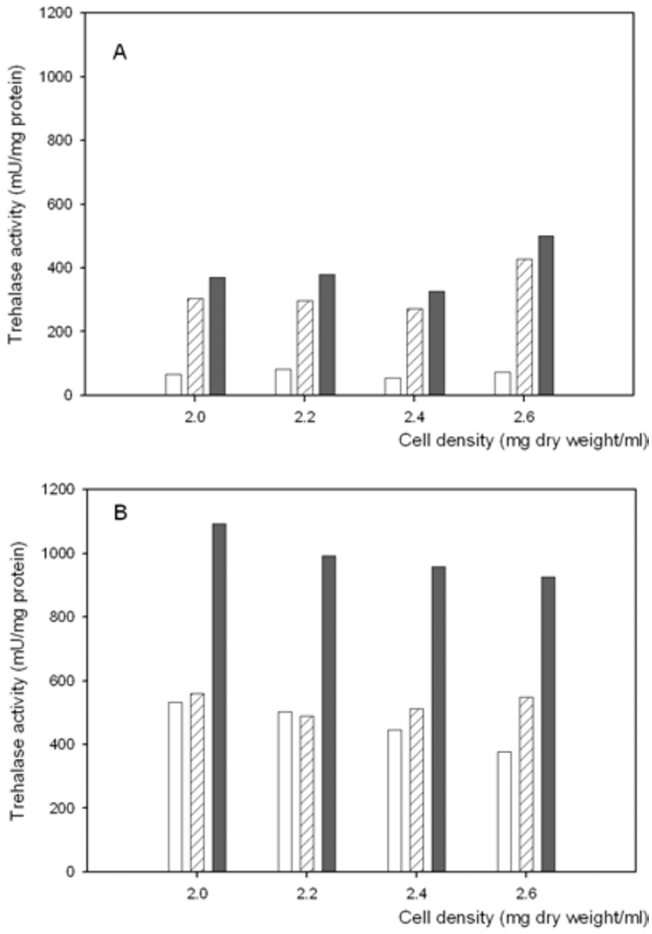

Figure 1
Basal, cryptic and total trehalase activities for the *YLR270w* (A) and *ylr270w* (B) strains. Cells were grown in YPD medium. Open bars represent basal trehalase activity; dashed bars, cryptic trehalase activity and filled bars, total trehalase activity (in vitro cAMP/ATP activated) determined in cells harvested at the indicated points.

cAMP concentration in this phase [8]. Activation by PKA reaches a maximum value at the onset of the transition phase of growth on glucose, from fermentative to oxidative metabolism. At this point, almost 80% of the enzyme is in a cryptic non-phosphorylated form [4]. We have previously shown that at this point trehalase remains in its inactive non-phosphorylated form by the action of an endogenous inhibitory protein [3]. Recently, a comprehensive two-hybrid analysis revealed a 41 KDa NTH1p binding protein encoded by *YLR270w* ORF with unknown function [5,6]. In order to clarify the mechanisms of trehalase down-regulation, the involvement of Ylr270p on trehalase modulation was studied. Here we have evaluated trehalase activation by PKA, as well as the expression of the *NTH1* gene promoter fused to the heterologous reporter *lacZ* gene in *ylr270w* background.

Our *in silico* analysis of the 5' upstream regions of the *YLR270w*, *TPS1* and *NTH1* genes revealed similar elements in their promoters. These data taken together with those found by an extensive data-mining over genomic micro-array databases point toward a coordinated expression of these genes.

Results and discussion

The first property observed for the Ylr270p was its ability to bind to trehalase therefore, we renamed it as Trehalase Associated Protein, Tap [9]. Later, Liu *et al.* [10] characterized the Ylr270p also as an enzyme with mRNA decapping activity, and introduced another name, yDcpS or *DCS1*.

DCS1p involvement in trehalase activity regulation was investigated by measuring basal and PKA-activated trehalase activities in both *DCS1* and *dcs1* cells grown on glucose and harvested at the transition phase leading to diauxie (Figure 1).

During the onset of diauxie (2.0–2.6 mg dry weight/ml) cells lacking the *DCS1* gene showed higher levels of trehalase activity than the control cells. This increase was due both to a higher basal activity in *dcs1* cells and to a higher total trehalase activity revealed after *in vitro* activation by PKA. In the control strain, basal trehalase activity corresponded to about 20% of total trehalase, which is in agreement with the results reported for other wild type strains [3,4,9]. However, we found that in the *dcs1* mutant this relation was about 50%. This fifty-fifty ratio between cryptic trehalase and basal trehalase is typically found at the exponential or stationary phase of growth when the trehalase inhibitory protein is reported as absent [3].

The trehalase inhibitory protein shows to be a Ca^{+2}/Calmodulin ligand [3] and possibly a substrate for the Ca^{+2}/Calmodulin protein kinase (CaM Kinase II) isozymes encoded by the *CMK1* and *CMK2* genes [9]. It has been proposed that this inhibitory protein acts as a mediator between the Ca^{+2} signal and trehalase activation by PKA. Indeed, when trehalase activity was measured in the presence of Ca^{+2} ions in crude extracts from *dcs1* mutants no activation occurred in contrast to the 2-fold activation seen in the control strain (unpublished results).

These results suggest that the product of the *YLR270w* ORF, Dcs1p, is involved in trehalase down regulation and could be the inhibitory protein reported by De Mesquita *et al* (1997). These findings are supported by the observation that the maximum expression of *DCS1* mRNA coincides with the lower levels of basal trehalase [3,7]. *DCS1*

Table 1: Effect of *ylr270w* mutation on the expression of the *NTH1-lacZ* reporter gene.

Strain	β-Galactosidase activity (Miller units)
Control (*YLR270w*)	26.3 ± 1.6
Mutant (*ylr270w*)	71.2 ± 4.8

Saccharomyces cerevisiae control and mutant strains transformed with pNL1 plasmid containing *NTH1-lacZ* fusion were grown in YNB with glucose as the carbon source as described in Materials and methods. See this same section for a description of plasmid and recipient yeast used. Data are reported as means ± SD of three independent transformants and three independent experiments.

mRNA expression levels were shown to be enhanced suddenly and transiently at the onset of diauxie [7], a condition where the trehalase inhibitory activity was found [3].

To determine whether the elevated total trehalase activity found in the *dcs1* mutant corresponded to a change in *NTH*1 expression, we evaluated *NTH1* expression by the activity of the *lacZ* reporter gene fused to 600 bp of *NTH1* promoter region [10].

As shown in table 1, the expression of *NTH1-lacZ* fusion gene was 2.5-fold higher in *dcs1* deleted than in control cells. These results raise the question whether Dcs1p could also be implicated, even in an indirect manner, in the regulation of *NTH1* transcription. Indeed, this should be the case since, recently, a homologous Dcs1 in *Schizosaccharomyces pombe*, was predominantly found in the nucleus albeit characterized as an mRNA binding protein capable of acting as a relatively poor translation inhibitor in this organism [12].

Over the years, an intriguing paradox remained unexplained: the concomitant trehalose accumulation and the enhanced trehalase expression under stress conditions. In fact *TPS1*, involved in trehalose biosynthesis, *NTH1* and *DCS1* exhibit a coordinated induction at diauxic growth [7].

These observations led us to investigate the presence of similar regulatory elements in the promoters of the *NTH1*, *TPS1* and *DCS1* genes in order to ascertain whether the trehalose/trehalase modulation by Dcs1p transcends the post-diauxic stress.

In our *in silico* analysis, 700 bp 5' upstream regions of the *NTH1*, *TPS1* and *YLR270w* genes were screened using the MatInspector v2.2 [13], FastM [14], TRES (Transcription Regulatory Element Search) [15], and SCPD (*Saccharomyces cerevisiae* Promoter Database) [16] algorithms searching for transcription factors motifs and consensus sequences annotated on the Transfac 5.0 database [17].

The results in table 2 show the similar elements shared in *NTH1*, *TPS1*, and *DCS1* promoters. All the putative elements found are involved in stress responses, caused by heat (STRE and HSTE), amino acid starvation (GCN4), and glicolysis/gluconeogenesis regulation (GCR1). The conserved motifs in these genes could be recognized by common factors and could mean a common regulatory program sharing similar expression profiles.

The approach to investigate the transcription profiles and characterize the *DCS1* gene in order to understand its role in the yeast metabolic scenery, was to mine microarrays databases. In these databases we searched for a change in the *YLR270w* ORF expression using a two-fold variation cutoff. Microarray experiments provide more information than what the authors can interpret alone. This "orphan" information offers clues about gene functions. The *YLR270w* ORF remained, until now, with an unknown function, and its expression data was never discussed.

To analyze the transcription profiles of *DCS1*, *NTH1*, and *TPS1* genes under different experimental conditions we used the Yeast Microarray Global Viewer (yMGV) [18], which allows direct comparison of the results from 1347 conditions from 75 different publications.

Heat stress at 37°C/20 minutes [19] led to a 6-fold increase in *DCS1* expression in wild type cells in contrast to a 2.0-fold increase in a *msn2*/*msn4* mutant under the same conditions. This result suggests that the STRE element found in the *DCS1* promoter region could be functional and mediated by Msn2 and Msn4 transcription factors. *NTH1* and *TPS1* genes showed similar profiles. These three genes behave similarly also in other stress conditions [20] showing to be co-regulated during changes in oxidation, pH, and osmolarity (figure 2B).

Starvations for amino acids, purines, as well as glucose limitation induce the synthesis of the Gcn4 transcription activator of amino acids biosynthetic genes, in multiple pathways. Computational searches of the yeast genome reveal that the Gcn4 binding site is present at the promoters of numerous genes not directly connected with amino acid biosynthesis [21].

Under nitrogen depletion [19], a significant increase in the expression of *DCS1*, *NTH1*, and *TPS1* genes can be observed after 8 h of nitrogen starvation. Corroborating these results, it has been demonstrated that these genes are induced up to 3.5-fold in wild type cells in response to starvation for histidine by treatment with 3-aminotriazole (3AT), a competitive inhibitor of His3p [21]. Therefore,

Table 2: Upstream elements shared in gene promoters of trehalose pathway.

	TAP		NTH1		TPS1	
	Position	Sequence	Position	Sequence	Position	Sequence
STRE	-518	AGGGG	-350	AGGGG	-472	AGGGG
			-342	AGGGG	-359	AGGGG
			-152	AGGGG	-305	AGGGG
					-278	AGGGG
					-249	AGGGG
					-239	AGGGG
HSTE	-515	GAATCTCC	-222	GAAAAATCC		
	-480	TTCTTTGAA				
	-470	GAAACATCC				
	-451	GAAAGTCC				
	-358	GAAGATCC				
GCN4	-693	TGACTT	-497	TGAATA	-626	TGATTT
	-553	TGATTT			-186	TGAGTA
	-496	TGAGTG			-134	TGATTA
	-487	TGAGTT				
	-414	TGACTG				
GCR1	-466	CATCC	-635	CATCC	-293	CATCC
	-354	CTTCC	-612	CTTCC		
	-329	CATCC	-413	CTTCC		
	-122	CATCC				
ADR1	-515	TCTCC	-650	TCTCC	-635	TCTCC
			-505	TCTCC	-316	TCTCC

Sequences were taken from the *Saccharomyces cerevisiae* Promoter Database.

when a *gcn4* mutant strain is treated with 3AT the expression profiles of *DCS1*, *NTH1*, and *TPS1* genes show only an increase of about 1.5 fold. These results reinforce the idea that the GCN4 elements found several times in *GCS1*, *NTH1*, and *TPS1* promoters (see table 2) could be involved in nitrogen limitation stress response in those genes. In addition, the treatment with the alkylating agent, methyl methane sulfonate (MMS), that induces *GCN4* translation [21] also induces *DCS1*, *NTH1* and *TPS1* expression [21].

Up to this day neutral trehalase has not been shown to be regulated by classical catabolite repression or derepression factors. However, *NTH1* expression increases around 4.0-fold in *gcr1* mutant strains after glucose addition to cells grown on lactate and glycerol. On the other hand, in the *GCR1* control strain a 0.3-fold decrease was found [22]. The same occurs with *DCS1* and *TPS1* genes [22]. These results bring support for the *GCR1* putative elements found in *DCS1*, *NTH1*, and *TPS1* promoters (table 2).

The yeast *gcr1* mutant grows on non-fermentative carbon sources at the same rate as wild type strains, however it exhibits a severe growth defect when grown in the presence of glucose, even when non-fermentable carbon sources are available [22]. The role of *Gcr1* as an activator is best understood in relation to genes coding for glycolytic enzymes. *Gcr1* is recruited to the UAS elements by Rap1 in order to activate its transcription. No changes were observed in *DCS1*, *NTH1*, and *TPS1* expression in rap1 mutant [23], what indicates that the Gcr1 factor regulation for these genes does not depend on Rap1. Therefore, we might speculate that, in the presence of glucose, *Gcr1* activates glycolytic genes with Rap1 and represses trehalose metabolism when either alone or complexed with another factor.

These results point toward trehalase as an alternative gluconeogenic enzyme forming glucose after stress conditions, when trehalose accumulated to protect the cell functions should be consumed as a carbon source in order to allow yeast cells to rapidly resume growth under favorable conditions.

Conclusions

Our results strongly indicate a central role for Dcs1p in inhibiting trehalase activation and preventing a wasteful futile cycle of synthesis and degradation of trehalose.

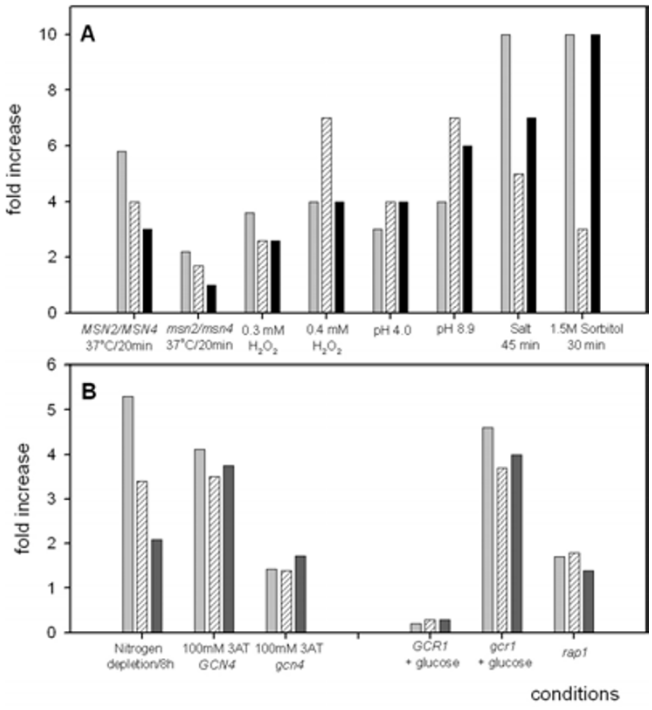

Figure 2
Comparison of *DCS1*, *NTH1*, and *TPS1* expression profiles. The 'alignment transcription profile for several genes' tool was used to search yMGV [18] for *DCS1* (gray bars), *NTH1* (striped bars) and *TPS1* (black bars) genes. The main similarities in their expression patterns are represented. (A) Induction by heat treatment [19]. (B) Induction by changes in oxidation, pH, and osmolarity [20]. (C) Induction by nitrogen starvation [19]. (D) Effect of *gcr1* mutation in the presence of glucose [22].

These conclusions provide a clear explanation for the above-mentioned paradox and broaden our understanding of metabolic fluxes in yeast cells.

Under stress conditions trehalose is accumulated in order to provide protection for membranes and proteins. During recovery from stress, trehalase is activated. On the other hand, should trehalose be required as a carbon source, activation of trehalase leads it through the gluconeogenic pathway.

Methods

Strains and media

The following *Saccharomyces cerevisiae* congenic strains were used: Y00000 (MATa; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*), Y05179 (MATa; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YLR270w::kanMX4*). Source: EUROSCARF, Institute for Microbiology – Johann Wolfgang Goethe-University; Frankfurt; Marie-Curie-Strasse 9; Building N250 - D-60439 Frankfurt – Germany.

Escherichia coli DH5α was used for plasmid manipulations.

Growth conditions

Yeast cells were grown either in a rich medium (YP) containing 1% yeast extract, 2% peptone or in a mineral medium (YNB, Difco yeast nitrogen base, with the necessary supplements); 2% glucose was used as carbon source. The pNL1 plasmid, a gift from C. Gancedo, was constructed inserting the *NTH1* promoter in frame to the *lacZ* gene in the Yep353 [11].

Enzymatic activity measurements

Yeast extracts were prepared by shaking with glass beads, and protein was determined by the standard method of Stickland [24].

Trehalase activity determination

The assay of basal neutral trehalase was performed in 50 mM maleate buffer pH 6.0, 100 mM trehalose in a total volume of 200 μL. After incubation for 15 min. at 30°C the reaction was stopped in boiling water bath (3 min.). Glucose was determined by the glucose oxidase-peroxidase method. To perform the activation of cryptic trehalase, 50 μL of the activation cocktail containing 2 mM ATP, 20 mM $MgCl_2$, 50 μM cAMP, 50 mM NaF and 5 mM theophylline prepared in 50 mM phosphate buffer, pH 7.5, was added to a suitable sample of the crude extract in a final volume of 100 μL. After incubation at 30°C for 15 min., the reaction was stopped by dilution with 400 μL of ice-cold 50 mM maleate buffer pH 6.0. Blanks were performed with omission of the activation cocktail. Basal trehalase activity is measured in cell free extracts prior to activation. Cryptic activity is the difference between totally activated trehalase and its basal activiy. One unit of trehalase is defined as the amount of enzyme that catalyses the hydrolysis of trehalose under the assay conditions giving rise to 1 μmol of glucose / minute.

β-galactosidase activity determination

For the β-galactosidase assay, cells were harvested and immediately frozen until use. Permeabilized cells were prepared with SDS and chloroform in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, pH7.0). β-galactosidase activity was determined by measuring 2-nitrophenyl-β-galactopyranoside hydrolysis [25].

Authors' contributions

JFM carried out the experimental and *in silico* studies, and prepared the draft of the manuscript. ADP and PSA participated in the design of the study, performed the critical

reading of the manuscript and coordination. All authors read and approved the final manuscript.

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